# METABOLISM OF ENDOSULFAN-ALPHA BY HUMAN LIVER MICROSOMES AND ITS UTILITY AS A SIMULTANEOUS *IN VITRO* PROBE FOR CYP2B6 AND CYP3A4

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# METABOLISM OF ENDOSULFAN-ALPHA BY HUMAN LIVER MICROSOMES AND ITS UTILITY AS A SIMULTANEOUS *IN VITRO* PROBE FOR CYP2B6 AND CYP3A4


## ABSTRACT:

Endosulfan-α was metabolized to a single metabolite, endosulfan sulfate, in pooled human liver microsomes ($K_m$ = 9.8 μM, $V_{max}$ = 178.5 pmol/mg/min). With the use of recombinant cytochrome P450 (rCYP) isoforms, we identified CYP2B6 ($K_m$ = 16.2 μM, $V_{max}$ = 11.4 nmol/nmol CYP/min) and CYP3A4 ($K_m$ = 14.4 μM, $V_{max}$ = 1.3 nmol/nmol CYP/min) as the primary enzymes catalyzing the metabolism of endosulfan-α, albeit CYP2B6 had an 8-fold higher intrinsic clearance rate ($CL_{int}$ = 0.70 μL/min/pmol CYP) than CYP3A4 ($CL_{int}$ = 0.09 μL/min/pmol CYP). Using 16 individual human liver microsomes (HLM), a strong correlation was observed with endosulfan sulfate formation and S-mephenytoin N-demethylase activity of CYP2B6 ($r^2$ = 0.79) while a moderate correlation with testosterone 6-β-hyroxylase activity of CYP3A4 ($r^2$ = 0.54) was observed. Ticlopidine (5 μM), a potent CYP2B6 inhibitor, and ketoconazole (10 μM), a selective CYP3A4 inhibitor, together inhibited approximately 90% of endosulfan-α metabolism in HLMs. Using six HLM samples, the percent total normalized rate (% TNR) was calculated to estimate the contribution of each CYP in the total metabolism of endosulfan-α. In five of the six HLMs used, the percent inhibition (% I) with ticlopidine and ketoconazole in the same incubation correlated with the combined % TNRs for CYP2B6 and CYP3A4. This study shows that endosulfan-α is metabolized by HLMs to a single metabolite,

endosulfan sulfate, and that it has potential use, in combination with inhibitors, as an *in vitro* probe for CYP2B6 and 3A4 catalytic activities.

## INTRODUCTION:

Endosulfan is an organochlorine pesticide and a contaminant at toxic superfund sites. It is currently applied as a broad spectrum insecticide to a variety of vegetables, fruits, cereal grains, and cotton (USEPA, 2002). Endosulfan is sold under the tradename of Thiodan and as a mixture of two isomers, namely 70% α- and 30% β-endosulfan (ATSDR, 2000). Endosulfan exposure has been shown to increase rodent liver weights and elevate microsomal enzyme levels (Gupta and Gupta, 1977). In mice, endosulfan exposure resulted in increased testosterone metabolism and clearance (Wilson and LeBlanc, 1998). Studies involving children suggest that long term environmental exposure to endosulfan causes delayed male sexual maturation and reduced testosterone levels (Saiyed et al., 2003). The mechanism by which endosulfan may exert these effects may involve its ability to activate the human pregnane X receptor (PXR) and induce the expression levels of cytochrome P450 (CYP or P450) enzymes, thereby increasing metabolic rates.

Prior to beginning an investigation of endosulfan's possible endocrine disrupting effects, we wished to examine its metabolic pathway in humans. To date, there are no published data on human metabolism of endosulfan nor on the possible contributions of CYP isoforms to its metabolism. Based on animal studies, a proposed metabolic pathway for endosulfan was published by the Agency for Toxic Substances and Disease Registry (ATSDR, 2000) and is shown in **Fig.1**. A study using cats reported the immediate presence of endosulfan sulfate in the

liver following intravenous administration of endosulfan (Khanna et al., 1979). In rats administered with a single oral dose of $^{14}$C-endosulfan, the metabolites sulfate, lactone, ether, and diol were detected in their feces five days later (Dorough et al., 1978). Analyses of human adipose tissue, placenta, umbilical cord serum, and milk samples demonstrated the presence of parent compound (α and β-endosulfan) and metabolites endosulfan sulfate, diol, lactone, and ether, albeit the sulfate was the predominant degradation product (Cerrillo et al., 2005).

The present study determined that endosulfan-α is metabolized to a single metabolite, endosulfan sulfate, in human liver microsomes and its metabolism is primarily mediated by CYP2B6 (at high efficiency) and CYP3A4 (at low efficiency). CYP2B6 is recognized to be expressed at only 3 to 5 % of total P450s in human livers (Gervot et al., 1999; Lang et al., 2001) while CYP3A4 is known as the most abundant P450 isoform, expressed at 20-60% of total P450s in human liver (Guengerich, 1995). The respective levels of CYP2B6 and CYP3A4 in human liver microsomes in combination with their strong affinity to endosulfan-α ($K_m$ = 16.2 and 14.4 μM, respectively) and their corresponding clearance rates of endosulfan ($CL_{int}$ = 0.70 and 0.09 μL/min/pmol CYP, respectively) presented a unique opportunity of investigating the potential of endosulfan-α to simultaneously probe for the *in vitro* catalytic activity of both CYP2B6 and 3A4.

## MATERIALS and METHODS:

**Chemicals.** Endosulfan-α, the predominant isomer (70 %) in commercial endosulfan, was used in the study of endosulfan metabolism. Endosulfan-α, endosulfan sulfate, endosulfan diol, endosulfan ether, and endosulfan lactone reference materials were purchased from ChemService (West Chester, PA). Stock solutions of endosulfan-α and metabolites were prepared in acetonitrile (ACN) and stored at -20 °C. $NADP^+$, glucose-6-phosphate, and glucose-6-phosphate dehydrogenase were purchased from Sigma-Aldrich (St. Louis, MO). HPLC grade water, ACN, EDTA, magnesium chloride, Tris, and all other chemicals not specified were purchased from Fisher Scientific (Pittsburgh, PA).

Ticlopidine, a potent mechanism-based chemical inhibitor to CYP2B6 (Richter et al., 2004), and ketoconazole, a selective chemical inhibitor to CYP3A4 (Baldwin et al., 1995) were purchased from Sigma-Aldrich (St. Louis, MO). Stock solutions of ticlopidine were prepared in distilled water and stored at room temperature. Ketoconazole was dissolved in methanol and stock solutions were stored at 4°C.

**Human Liver Microsomes (HLMs) and CYP isoforms.** Pooled HLMs (20 mg/mL) and 16 selected individual HLMs (20 mg/mL each) were purchased from BD Biosciences. The individual HLMs chosen for this study were representative of the levels of S-mephenytoin N-demethylase activity of CYP2B6 as follows: (Low) HG32, HG95, HH47, HG74, HK37; (Mid) HG43, HG93, HH18, HK25, HH101, HG3; and (High) HH13, HG89, HG64, HG112, HG42. Human recombinant CYP (rCYP) and recombinant flavin monooxygenase (rFMO) isoforms expressed in baculovirus-infected insect cells (supersomes) were also purchased from BD Biosciences.

**Metabolism assays.** Preliminary studies were performed to determine the times and HLM protein concentrations which produced a linear metabolic rate for 50 μM of endosulfan-α. Endosulfan sulfate formation was linear from 0.05 to 0.25 mg/mL protein and from 5 to 60 min of incubation. The solvent effects of dimethyl sulfoxide (DMSO), acetone, acetonitrile (ACN), methanol, ethanol, and isopropanol at 1% solvent concentration were also tested on endosulfan-α metabolism. There were no differences in the rates of endosulfan sulfate formation among the different solvents, with the exception of isopropanol which slightly inhibited formation of endosulfan sulfate (data not shown).

Based on the results of initial studies, 20 μM endosulfan-α substrate concentration dissolved in ACN, 0.25 mg/mL protein concentration, and 30 min incubation time were used for subsequent metabolism assays, unless otherwise stated. Metabolism assays with HLMs utilized 100 mM potassium phosphate buffer (pH 7.4). Metabolism with rCYPs and rFMOs utilized the following buffers as recommended by BD Biosciences: 100 mM potassium phosphate (pH 7.4) for 1A1, 1A2, 3A4, 3A7, 2D6*1, 3A5, and SF9 insect control; 50 mM potassium phosphate (pH 7.4) for 2B6, 2C8, 2C19, and 2E1; 100 mM tris (pH 7.4) for 2C9*1, 2C18, and 4A11; 50 mM tris (pH 7.4) for 2A6; and 50 mM glycine (pH 9.5) for FMOs 1, 3, and 5. All buffers contained 3.3 mM $MgCl_2$ and 1mM EDTA.

A pre-incubation mixture of endosulfan-α (20 μM), HLMs (0.25 mg/mL) or rCYP isoforms (12.5 pmol), and buffer was prepared in 1.5 mL microcentrifuge tubes. This mixture was pre-incubated for 3 min at 37°C waterbath with minimal agitation. NADPH-regenerating system (final concentration of 0.25 mM $NADP^+$, 2.5 mM glucose-6-phosphate, and 2 U/mL glucose-6-phosphate-dehydrogenase) was added to initiate reaction. The final assay volume was 250 μL. Reactions were carried out for 30 min and terminated with 250 μL cold ACN, followed by pulse-

vortexing. Samples were centrifuged at 16,000 rpm for 5 minutes and supernatants were analyzed in HPLC, as described in the HPLC analysis section below.

**Inhibition studies.** Protocols for CYP2B6 and CYP3A4 inhibition by ticlopidine and ketoconazole utilized methods previously established by Richter et al (2004) and Nomeir et al. (2001), respectively. In the case of ticlopidine, a mechanism-based inhibitor of CYP2B6, a 3 min pre-incubation at 37°C of ticlopidine (5 μM) with HLMs (100 μg) or rCYPs (5 pmol) in 50 mM potassium phosphate buffer (with 3.3 mM $MgCl_2$ and 1mM EDTA) in combination with an NADPH regenerating system (final concentration of 0.5 mM $NADP^+$, 5 mM glucose-6-phosphate, and 4 U/mL glucose-6-phosphate dehydrogenase), occurred prior to the addition of endosulfan-α (20 μM). In the case of ketoconazole, endosulfan-α (20 μM) and ketoconazole (10 μM) were pre-incubated along with 100 μg HLMs or 5 pmol rCYP in 50 mM potassium phosphate buffer for 3 min at 37°C prior to the addition of the NADPH regenerating system (final concentration of 0.25 mM $NADP^+$, 2.5 mM glucose-6-phosphate, and 2 U/mL glucose-6-phosphate-dehydrogenase). In both cases, final reaction volumes were 250 μL and reactions were terminated by the addition of 250 μL cold ACN and processed as previously described.

**High-performance liquid chromatography (HPLC) analysis.** Metabolite formation was analyzed with a Shimadzu HPLC system consisting of an auto-injector (SIL-10AD VP), two pumps (LC-10AT), and a UV detector (SPD-10A VP). Endosulfan-α and metabolites were separated by a Gemini C18 column, 5μm, 100 x 4.6 mm (Phenomenex) and identified with direct injection of reference compounds. The mobile phase for pump A consisted of 99% water and 1% phosphoric acid (pH 2.0) and for pump B, 100% ACN. The flow rate was 1 mL/min. A gradient methodology was used as follows: 0 to 3 minutes (60% ACN), 3 to 16 minutes (60-90% ACN), 16 to 19 minutes (90-60% ACN), and 19 to 20 minutes (60% ACN). The injection

volume was 50 μL and solutes were detected at 213 nm. Under these conditions, the retention times for endosulfan-α and endosulfan sulfate were 12.4 and 8.9 minutes, respectively. Endosulfan-α and endosulfan sulfate peaks were quantified with calibration curves constructed from known concentrations of reference materials. The detection limit for endosulfan sulfate following the US Environmental Protection Agency's method detection limit procedure was 0.04 uM (CFR, 2006).

**Data Analyses.** Michaelis-Menten and Eadie-Hofstee plots were generated using Sigma Plot Enzyme Kinetics Module (Chicago, IL). Enzyme kinetic parameters $K_m$ and $V_{max}$ were determined using non-linear regression analysis with the Sigma Plot software.

Correlations of endosulfan sulfate formation with each CYP-specific catalytic activity or CYP contents were calculated with simple linear regression using the web-based Statcrunch program (www.statcrunch.com). $p<0.05$ was considered statistically significant.

To estimate the contributions of different CYP isoforms to metabolism of endosulfan-α, percent total normalized rates (% TNR) were calculated using the method described by (Rodrigues, 1999). Briefly, metabolite formation rate (pmol/min/pmol rCYP) obtained from rCYP metabolism of the compound of interest is multiplied by the immunoquantified CYP content (pmol nCYP/mg) in native human liver microsomes, yielding the "normalized rate" (NR) expressed in pmol/min/mg microsomes. The NRs for each CYP involved in the metabolism of the compound of interest is summed up as the "total normalized rate" (TNR) (Rodrigues, 1999). The % TNR for each CYP was then calculated according to the following equation.

$$\%\,TNR = \frac{NR}{TNR} \times 100 = \frac{pmol/\min/\,pmol\,rCYP \times pmol\,nCYP/mg}{\sum(pmol/\min/\,pmol\,rCYP \times pmol\,nCYP/mg)} \times 100$$

## RESULTS:

**Metabolism of endosulfan-α.** Endosulfan-α at 50 μM concentration was metabolized by pooled human liver microsomes (pHLM) to a single metabolite, endosulfan sulfate. **Fig. 2** shows a representative HPLC chromatogram of this metabolism assay. The retention times for endosulfan-α and endosulfan sulfate were 12.23 and 8.73 min, respectively, in a 20 min HPLC run.

**Cytochrome P450 screening.** Cytochrome P450 (CYP) and flavin-containing monooxygenase (FMO) contributions to metabolism of endosulfan-α (20 uM) were investigated using 14 rCYPs and 3 rFMO commercially available human isoforms. Recombinant CYP2B6 predominantly mediated the formation of endosulfan sulfate by 8-fold (at 6.9 nmol/min/nmol CYP) over the next isoform (CYP3A4) with the next highest metabolite formation rate (at 0.8 nmol/min/nmol CYP). CYPs 2C18, 2C19, 2C9*1, and 3A7 also showed metabolic activity, but at negligible levels (**Fig. 3**).

**Kinetics of endosulfan-α metabolism.** The kinetic parameters $K_m$ and $V_{max}$ were determined by incubating endosulfan-α (0.78-100 μM) with pHLM (0.25 mg/mL), r-CYP2B6 or r-CYP3A4 (12.5 pmol). Calculated apparent $K_m$, $V_{max,}$ and $CL_{int}$ are shown in **Table 1**.

The respective Michaelis-Menten (M-M) and Eadie-Hofstee plots of endosulfan-α metabolism by pHLM, r-CYP2B6 and r-CYP3A4 are shown in **Fig. 4A-C**. The M-M plot show a hyperbolic curve, indicating saturation of metabolite formation over the substrate concentration range used and suggesting that the data obeyed M-M kinetics. The Eadie-Hofstee plots were linear, indicating either involvement of one enzyme or of more than one enzyme with similar affinity (Ward et al., 2003), and with a slight hook at the bottom end of the curve, suggesting allosteric activation (Faucette et al., 2000).

**Correlation of endosulfan sulfate formation with specific CYP contents and selective CYP activities.** Endosulfan-α metabolism was conducted in 16 individual HLMs. Results of correlations between selective CYP activities from these 16 individual HLMs and specific CYP contents (of a subgroup of 8 HLMs with immunoquantified CYP contents from BD Biosciences) are shown in **Table 2**. Strong correlations were evident between endosulfan sulfate formation and CYP2B6 and 3A4 contents as determined by immunoquantitation ($r^2 = 0.86$ and 0.81, respectively). Likewise, there was a strong correlation between endosulfan sulfate formation and S-mephenytoin N-demethylase activity of CYP2B6 ($r^2 = 0.79$). A less significant correlation was found with testosterone 6-β-hyroxylase activity of CYP3A4 ($r^2 = 0.54$). No significant correlations were found for the other selective CYP activities and respective contents.

**Inhibition of endosulfan-α metabolism by ticlopidine and ketoconazole, selective chemical inhibitors for CYP2B6 and 3A4, respectively.** Initially, the optimal concentrations of ticlopidine and ketoconazole needed to obtain maximal inhibition of endosulfan sulfate formation were tested in rCYP2B6 and rCYP3A4. Results of these experiments are shown in **Fig. 5A-B**. It was determined that 5 uM ticlopidine and 10 uM ketoconazole were optimal for subsequent inhibition studies.

Results of inhibition of endosulfan sulfate formation with ticlopidine (5 μM) or/and ketoconazole (10 μM) are shown in **Table 3.** Six individual HLMs were chosen for these studies, based on available immunoquantified CYP contents data supplied by manufacturer. These individual HLMs also represented various ranges of CYP contents (see **Table 4**). Inhibition of endosulfan sulfate formation by ketoconazole among the six individuals varied from 9 to 38%, implicating varying levels of CYP3A4 among these individuals. Similarly, the range of

CYP2B6 involvement varied from 33 to 80%. The results show that inhibition of endosulfan metabolism with ketoconazole and ticlopidine were generally additive in all six HLMs.

**Percent Total Normalized Rate (% TNR).** % TNR was calculated to verify the percent inhibition (% I) results from this study (**Table 4**). % TNR obtained from rCYPs can be directly related to % I obtained with native HLMs (Rodrigues, 1999).

The % I from the combined incubation with ketoconazole and ticlopidine matched the sum of % TNRs of CYP2B6 and 3A4 in the metabolism of endosulfan-α in five of the six HLMs in this study (see **Table 5**).

## DISCUSSION:

In the present study, we found endosulfate sulfate as the only metabolite of endosulfan from incubations with HLMs. In mice exposed to a single dose of $^{14}$C-endosulfan, endosulfan sulfate concentrations were elevated in the liver, intestine, and visceral fat after 24 hours (Deema et al., 1966). A study in rats administered a single oral dose of $^{14}$C-endosulfan showed that the endosulfan metabolites diol, sulfate, lactone, and ether were found in the feces five days later (Dorough et al., 1978). A recent study conducted in Spain where endosulfan is commonly used identified parent endosulfan and metabolites diol, sulfate, lactone and ether in adipose tissues, placenta, cord blood and human milk (Cerrillo et al., 2005). These findings coupled with results of our study suggest that the diol, ether, and lactone metabolites may be the result of metabolic processes beyond those occurring in human liver microsomes.

Our kinetic studies with human liver microsomes as well as with CYP isoforms 2B6 and 3A4 produced monophasic Eadie-Hofstee plots, suggesting that endosulfan-α is metabolized either by one enzyme or by more than one enzyme with similar $K_m$. A survey of 14 CYP isoforms demonstrated significant metabolism by CYP2B6, followed by 3A4, members of the 2C family and 3A7. Of these isoforms, CYP2B6 and 3A4 are likely to have the greatest impact based upon activity levels and relative abundance. Although CYP2C18 may be similar to CYP3A4 in capacity to metabolize endosulfan, it is poorly expressed in human livers (Goldstein, 2001). Our kinetic studies demonstrated that CYP2B6 and CYP3A4 share similar binding affinities (Km of 16.2 and 14.4 μM, respectively) but vary significantly in maximum velocity. The resulting difference in clearance of endosulfan sulfate demonstrates that CYP2B6 is 8-fold more efficient than CYP3A4 in catalyzing the metabolism of endosulfan-α (see **Table 1**).

Initial inhibition studies utilizing monoclonal antibodies to CYP2B6 and 3A4 were abandoned due to their poor ability to inhibit endosulfan sulfate formation in the recombinant CYP isoforms (less than 30%; data not shown). This suggests that these monoclonal antibodies, although specific in inhibiting the metabolism of some substrates, may not be optimal inhibitors for endosulfan or other substrates. Hence, we used ticlopidine and ketoconazole, selective chemical inhibitors for CYP2B6 and 3A4 respectively, to characterize the contributions of these isoforms to endosulfan-α metabolism. At the concentrations used, these inhibitors did not significantly inhibit the activity of the other isoform examined (**Fig 5**). It is of interest that in the six HLMs examined, the combined use of ketoconazole and ticlopidine resulted in inhibition of endosulfan sulfate formation which was generally similar to the results obtained with each inhibitor alone. For four individuals, the combined inhibition of CYP2B6 and 3A4 yielded values from 85 to 92%, yet two individuals retained significant ability to metabolize endosulfan following inhibition (HK23 and HG93 with 57 and 76% inhibition, respectively). To further explore the possibility that other CYPs were involved in metabolism for these individuals, the total normalized rates of metabolism for the CYP isoforms identified by screening efforts were investigated.

The % I from the combined incubation with ketoconazole and ticlopidine corresponded well with the combined % TNRs of CYP2B6 and 3A4 (**Table 5**) in the metabolism of endosulfan-α in five of the six HLMs in this study. It seemed that with HK23, there was a significantly lower % inhibition of endosulfan-α metabolism by CYP2B6 (as demonstrated by % I with ticlopidine) when compared to the metabolic contribution of CYP2B6 as predicted by % TNR. This decreased inhibition of CYP2B6 activity in HK23 may be due to a CYP2B6 polymorphism. This is supported by a study in which a 26 % decrease was seen in N, N', N''-triethylene-

thiophosphoramide (tTEPA) inactivation of O-deethylation of 7-ethoxy-4-(trifluoromethyl)coumarin (7-EFC) in mutant CYP2B6 compared to wildtype 2B6 (Bumpus et al., 2005). It is now known that CYP2B6 polymorphisms are common in Caucasians and that CYP2B6 is one of the most polymorphic human P450s (Lang et al., 2001).

A number of substrate probes for CYP2B6 have been reported in the literature, including 7-ethoxy-4-trifluoromethylcoumarin (Code et al., 1997), cyclophosphamide and ifosfamide (Huang et al., 2000), S-mephenytoin (Heyn et al., 1996; Ko et al., 1998), bupropion (Faucette et al., 2000; Hesse et al., 2000), and efavirenz (Ward et al., 2003). The known substrate probes for CYP3A4 include testosterone, midazolam, nifedipine, and erythromycin (Yuan et al., 2002). The use of one substrate to simultaneously probe for the *in vitro* catalytic activity of CYP2B6 and CYP3A4 would be very advantageous. Based on the results of our inhibition studies, endosulfan-α appears to be a strong candidate for this role.

In conclusion, endosulfan-α is metabolized to a single metabolite, endosulfan sulfate, by HLMs. This metabolism is primarily mediated by CYP2B6 and CYP3A4. The strategies employed to demonstrate this were: 1) endosulfan-α metabolism by rCYPs, 2) correlation studies of endosulfan sulfate formation and CYP-selective activities or CYP immunoquantified contents in individual HLMs, and 3) inhibition studies using CYP2B6 and CYP3A4 selective chemical inhibitors. In addition, endosulfan-α may be utilized to simultaneously probe for the in-vitro catalytic activities of CYP2B6 and CYP3A4. Finally, endosulfan's endocrine disrupting effects and mechanisms inducing microsomal enzyme activity are currently under investigation.

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**Footnotes:**

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**Figure legends:**

**Fig. 1**. The proposed metabolic pathway for endosulfan based on animal studies, as published by ATSDR, 2000, was modified to show that human CYP2B6 and CYP3A4 primarily catalyzes the metabolism of endosulfan-α to endosulfan sulfate, the only metabolite detected in the present study.

**Fig. 2**. A representative HPLC chromatogram of endosulfan-α metabolism to endosulfan sulfate, the lone metabolite detected in incubations with human liver microsomes. The three peaks towards the end of the chromatogram were determined to be contributions from human liver microsomes.

**Fig. 3**. Rates of endosulfan sulfate formation from endosulfan-α (20 μM) by 14 recombinant cytochrome P450s (rCYPs) and 3 recombinant flavin monoxygenase (r-FMO) isoforms. Data shown are the means of two independent determinations.

**Fig. 4**. Velocity of endosulfan sulfate formation versus endosulfan-α concentration in human liver microsomes (A), recombinant CYP2B6 (B), and recombinant CYP3A4 (C). Each point represents the mean of three independent measures.

**Fig. 5**. Inhibition of endosulfan sulfate formation in rCYP2B6 and rCYP3A4 by (A) ketoconazole (0-10 μM) and (B) ticlopidine (0-10 μM). Each point represents the mean of two independent measures.

**Fig. 1.**

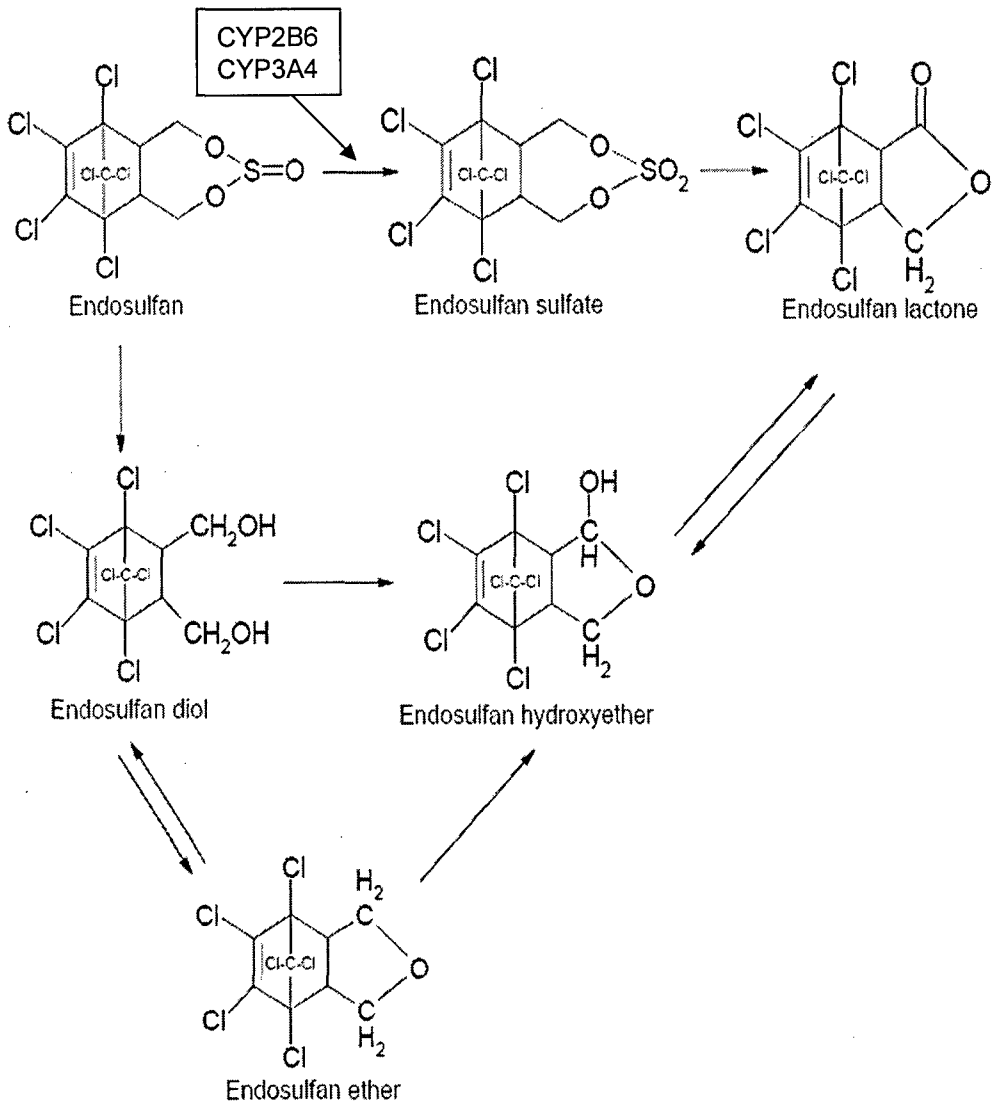

CYP2B6
CYP3A4
Endosulfan
Endosulfan sulfate
Endosulfan lactone
Endosulfan diol
Endosulfan hydroxyether
Endosulfan ether

Fig. 2.

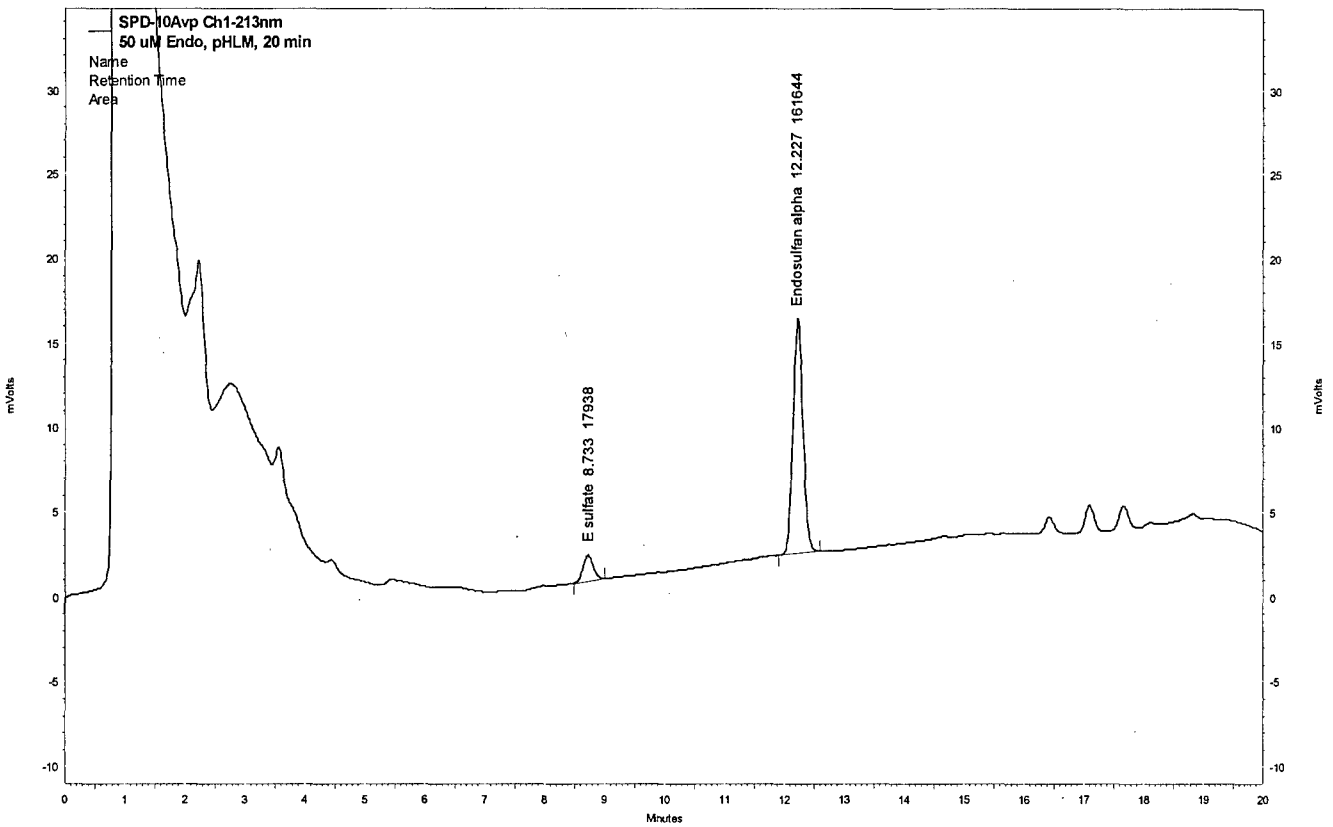

SPD-10Avp Ch1-213nm
50 uM Endo, pHLM, 20 min
Name
Retention Time
Area
E sulfate 8.733 17938
Endosulfan alpha 12.227 161644
mVolts
Minutes

Fig. 3.

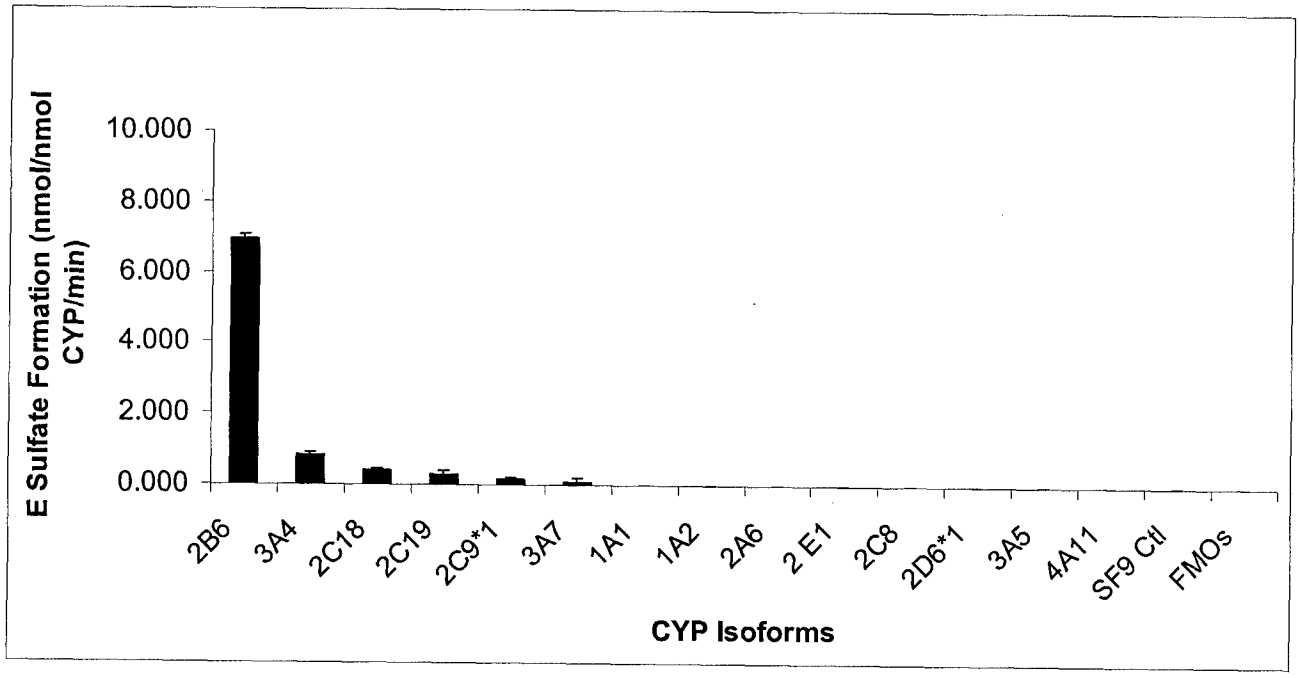

E Sulfate Formation (nmol/nmol CYP/min)
10.000
8.000
6.000
4.000
2.000
0.000
2B6
3A4
2C18
2C19
2C9*1
3A7
1A1
1A2
2A6
2 E1
2C8
2D6*1
3A5
4A11
SF9 Cll
FMOs
CYP Isoforms

**Fig. 4**

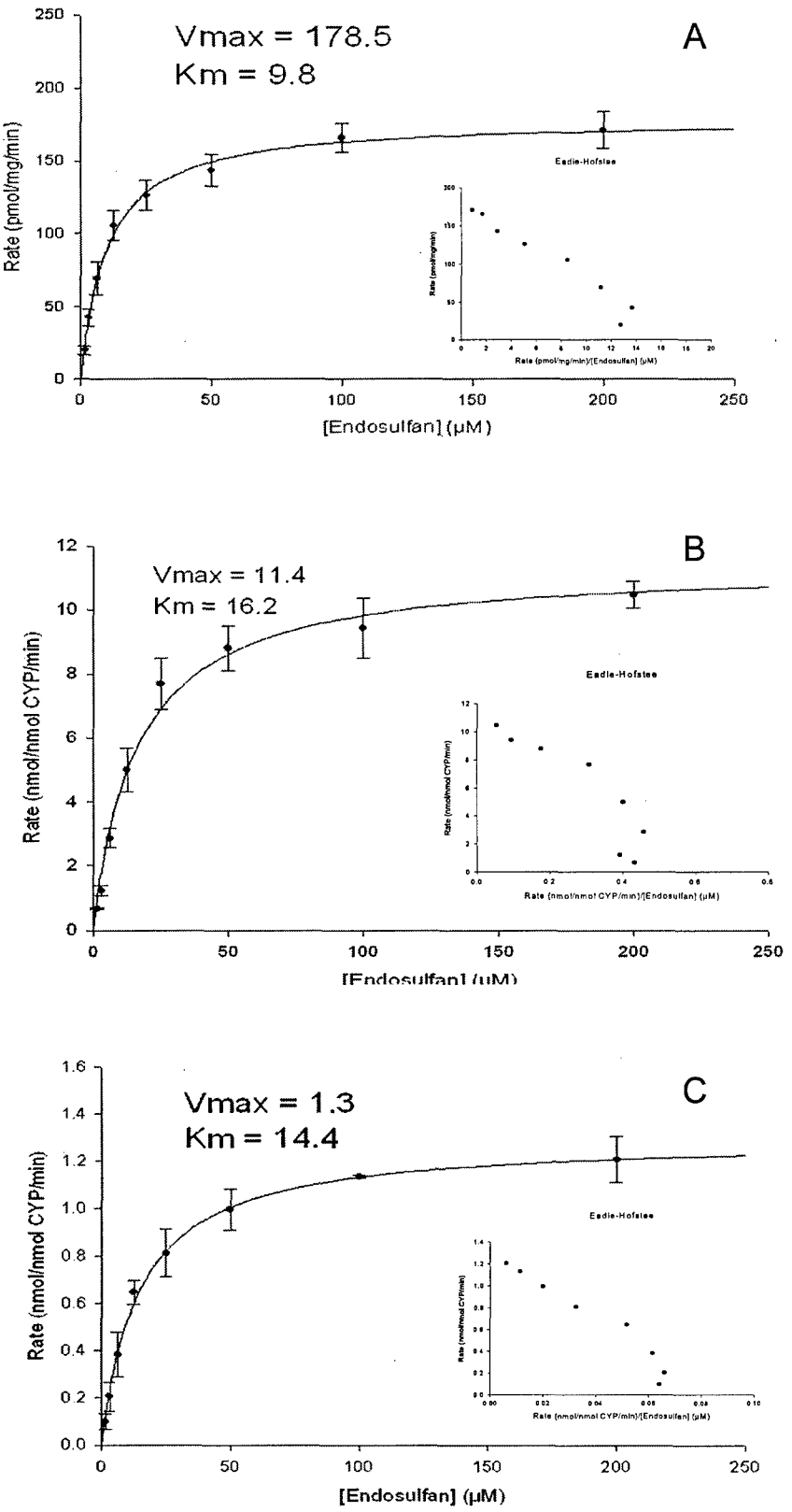
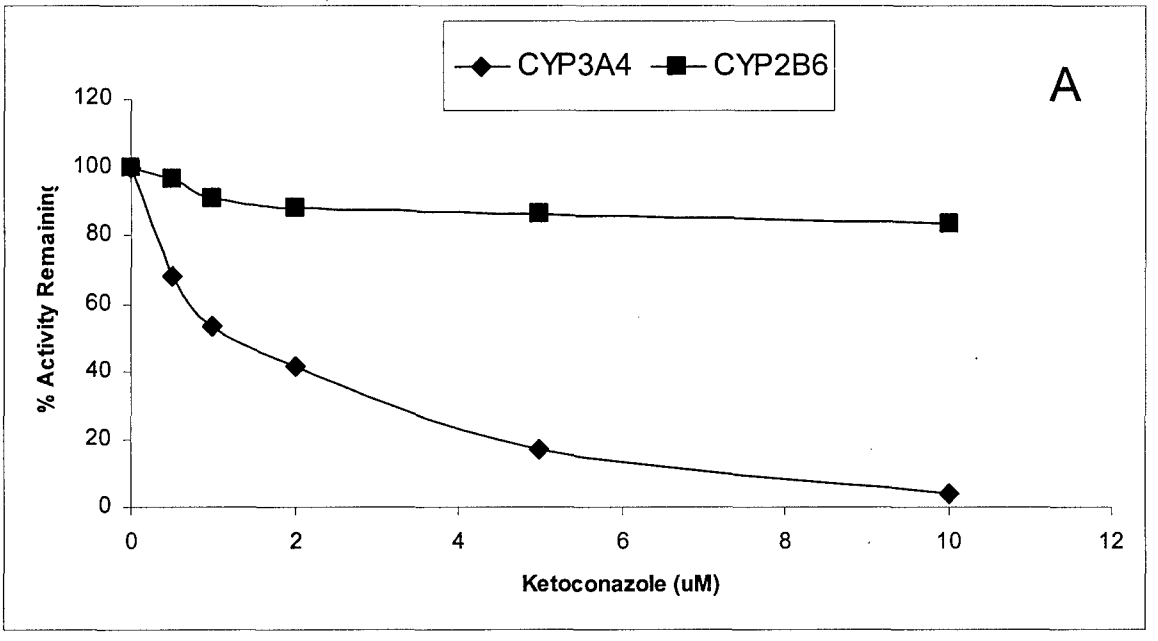
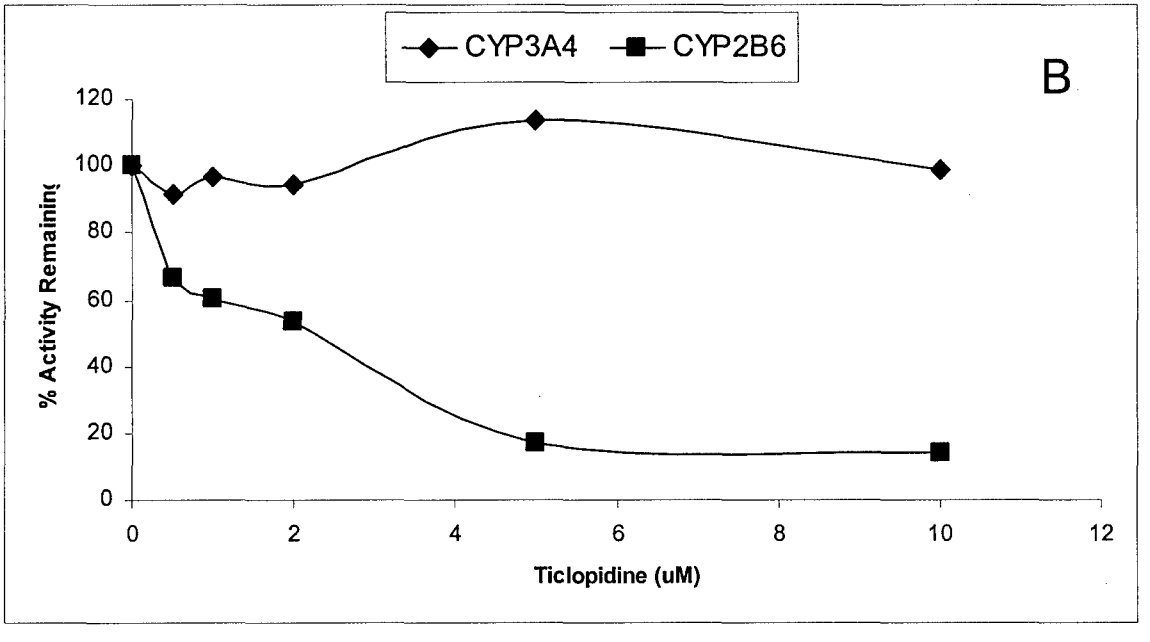

A
Vmax = 178.5
Km = 9.8
Rate (pmol/mg/min)
[Endosulfan] (μM)
Eadie-Hofstee
Rate (pmol/mg/min)/[Endosulfan] (μM)
B
Vmax = 11.4
Km = 16.2
Rate (nmol/nmol CYP/min)
[Endosulfan] (μM)
Eadie-Hofstee
Rate (nmol/nmol CYP/min)/[Endosulfan] (μM)
C
Vmax = 1.3
Km = 14.4
Rate (nmol/nmol CYP/min)
[Endosulfan] (μM)
Eadie-Hofstee
Rate (nmol/nmol CYP/min)/[Endosulfan] (μM)

Fig. 5


CYP3A4
CYP2B6
A
120
100
80
60
40
20
0
% Activity Remainin
0
2
4
6
8
10
12
Ketoconazole (uM)


CYP3A4
CYP2B6
B
120
100
80
60
40
20
0
% Activity Remainin
0
2
4
6
8
10
12
Ticlopidine (uM)

## TABLE 1

**Kinetic parameters of endosulfan-α metabolism in pooled human liver microsomes (pHLM), recombinant CYP2B6 and 3A4**

| HLMs or CYP | $K_m$ (μM) | $V_{max}$ | $Cl_{int}$ |
|---|---|---|---|
| pHLM | 9.8 | 178.5[a] | 18.20[c] |
| CYP2B6 | 16.2 | 11.4[b] | 0.70[d] |
| CYP3A4 | 14.4 | 1.3 | 0.09 |

[a] *Vmax* expressed in pmol/min/mg protein for pHLM and in pmol/min/pmol CYP for CYP2B6 and 3A4

[b] Vmax expressed in pmol/min/pmol CYP for CYP2B6 and 3A4

[c] Intrinsic clearance (Vmax/Km) expressed in μL/min/mg protein for pHLM

[d] Intrinsic clearance (Vmax/Km) expressed in μL/min/pmol CYP for CYP2B6 and 3A4

## TABLE 2

### Correlation of rates of endosulfan sulfate formation and CYP-selective activities or CYP-specific contents

Rate of endosulfan sulfate formation from 50 μM endosulfan-α was determined in 16 individual human liver microsomes (HLMs). Determinations were done in two independent experiments. The rates of CYP-selective activities and CYP-specific contents for these 16 HLMs were supplied by the manufacturer. Statistical analyses were done using simple non-linear regression in the web-based StatCrunch program. $p < 0.05$ was considered statistically significant.

| Rates of CYP-selective activities (pmol/min/mg) or CYP content (pmol/mg) | Rate of Endosulfan sulfate formation (pmol/min/mg) | |
|---|---|---|
| | $r^2$ | $p$ |
| s-Mephenytoin N-demethylase activity of 2B6 | 0.79 | <0.0001 |
| Testosterone 6-B-hydroxylase activity of 3A4 | 0.55 | 0.001 |
| Diclofenac-4-hydroxylase activity of 2C9 | 0.04 | 0.460 |
| s-Mephenytoin 4-hyroxylase activity of 2C19 | 0.01 | 0.743 |
| 2B6 content | 0.86 | 0.0008 |
| 3A4 content | 0.81 | 0.002 |
| 2C9 content | 0.42 | 0.167 |
| 2C19 content | 0.01 | 0.571 |

## Table 3

### Inhibition of E sulfate formation in HLMs by Ketoconazole and Ticlopinid

Inhibitors ketoconazole (10 uM) and ticlopidine (5 uM) were used alone and combined for metabolism of endosulfan-α in six individual human liver microsomes (HLMs). Data are means of two independent measurements.

| | % Inhibition of Endosulfan sulfate formation | | | | | |
|---|---|---|---|---|---|---|
| **Inhibitor** | **HG3** | **HG112** | **HG42** | **HG43** | **HG93** | **HK23** |
| **10 uM KTZ** | 23.8 ± 0.6 | 20.5[a] | 8.6 ± 1.5 | 34.9 ± 4.7 | 37.6 ± 8.8 | 36.0 ± 0.2 |
| **5 uM TCL** | 67.8 ± 2.6 | 64.4 ± 0.5 | 79.2 ± 3.0 | 57.0 ± 0.4 | 38.6 ± 3.3 | 33.0 ± 4.8 |
| **5 uM TCL + 10 uM KTZ** | 92.3 ± 0.4 | 88.0 ± 1.3 | 91.5 ± 2.4 | 85.2 ± 0.6 | 75.6 ± 6.2 | 57.0 ± 0.6 |

[a]No replicate for this measurement due to insufficient HLM HG112 sample.

## TABLE 4

### Comparison between % Total Normalized Rates (% TNR) and % Inhibition (% I) in three human liver microsomes (HLMs)

% TNR was calculated according to Rodrigues et al, 1999. % I for CYP2B6 was determined with the use of ticlopidine (5 μM) and for CYP3A4 with ketoconazole (10 μM).

| HLMs | Recombinant CYP (rCYP) | E sulfate formation rate[a] in rCYP | CYP content[b] in native HLMs | Normalized Rate | % TNR | % I |
|---|---|---|---|---|---|---|
| HG42 | 2B6 | 9.42 | 53 | 499.37 | 64.8 | 79.2 |
| | 2C9 | 0.34 | 80 | 24.12 | 3.5 | ND[c] |
| | 2C19 | 0.32 | 6 | 1.89 | 0.2 | ND |
| | 3A4 | 0.78 | 310 | 242.73 | 31.5 | 8.6 |
| HG112 | 2B6 | 9.42 | 47 | 442.83 | 58.8 | 64.4 |
| | 2C9 | 0.34 | 87 | 29.49 | 3.9 | ND |
| | 2C19 | 0.32 | 72 | 22.68 | 3.0 | ND |
| | 3A4 | 0.78 | 330 | 258.39 | 34.3 | 20.5 |
| HG3 | 2B6 | 9.42 | 18 | 169.60 | 65.0 | 67.8 |
| | 2C9 | 0.34 | 42 | 14.24 | 5..4 | ND |
| | 2C19 | 0.32 | 9 | 2.84 | 1.1 | ND |
| | 3A4 | 0.78 | 95 | 74.38 | 28.5 | 23.8 |
| HK23 | 2B6 | 9.42 | 7 | 65.95 | 42.1 | 33.0 |
| | 2C9 | 0.34 | 56 | 18.98 | 12.1 | ND |
| | 2C19 | 0.32 | 17 | 5.36 | 3.4 | ND |
| | 3A4 | 0.78 | 85 | 66.56 | 42.4 | 36.0 |
| HG43 | 2B6 | 9.42 | 4 | 37.69 | 26.3 | 57.0 |
| | 2C9 | 0.34 | 51 | 17.29 | 12.1 | ND |
| | 2C19 | 0.32 | 47 | 14.81 | 10.3 | ND |
| | 3A4 | 0.78 | 94 | 73.60 | 51.3 | 34.9 |
| HG93 | 2B6 | 9.42 | 18 | 169.60 | 69.0 | 38.6 |
| | 2C9 | 0.34 | 51 | 17.29 | 7.3 | ND |
| | 2C19 | 0.32 | 49 | 15.44 | 6.7 | ND |
| | 3A4 | 0.78 | 52 | 40.72 | 17.0 | 37.6 |

[a] Rates in pmol/min/pmol rCYP
[b] Immunoquantified CYP contents in pmol/min/mg protein
[c] Not determined.

## TABLE 5

### Sum of CYP2B6 and 3A4 % TNRs vs. % I with Ketoconazole and Ticlopidine

Comparison between the sum of % Total Normalized Rates (%TNRs) of CYP2B6 and CYP3A4 in the metabolism of Endosulfan-α and % Inhibition (% I) with ketoconazole and ticlopidine in the same incubation. With the exception of HK23, the other 5 HLMs had matching % TNR and % I.

| HLM | % TNR | % I |
|---|---|---|
| HG3 | 94 | 92 |
| HG112 | 93 | 88 |
| HG42 | 96 | 92 |
| HK23 | 84 | 57 |
| HG43 | 78 | 85 |
| HG93 | 86 | 76 |